

Effect of catecholamines on intracellular cytokine synthesis in human monocytes

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ABSTRACT. Proinflammatory cytokines produced by monocytes, like Interleukin-6 (IL-6), Interleukin-8 (IL-8), and tumor necrosis factor (TNF- α) are known for their pivotal role in the initiation of the inflammatory response following cardiopulmonary bypass (CPB). Catecholamines like epinephrine (Epi) and norepinephrine (Nor) are often necessary to stabilize the cardiac function in the early postoperative period and may influence the cytokine expression in monocytes.

In this study we investigated the effects of Epi and Nor on IL-6, IL-8 and TNF- α expression in human monocytes stimulated with lipopolysaccharide (LPS) in whole blood, analyzed intracellularly by flow cytometry. Kinetics of intracellular proinflammatory cytokine production and LPS ED₅₀ were obtained. To simulate different stages of inflammation *in vivo*, varying concentrations of LPS (0.2 ng/ml, 1 ng/ml and 10 ng/ml) were used for stimulation. After a stimulation with LPS TNF- α was the first produced cytokine, followed by IL-8 and IL-6. All cytokines peaked from 3 h to 6 h. Epi and Nor had comparable effects on the expression of IL-6, IL-8 and TNF- α in monocytes. Both inhibited IL-6 and TNF- α expression in a concentration dependent manner whereas IL-8 expression remained unchanged.

We conclude that monocytes are targets for Epi and Nor concerning their cytokine expression. The inhibiting effects of Nor and Epi were almost identical for all cytokines. Cytokine expression was affected most at low LPS concentrations.

Keywords: proinflammatory cytokines, monocytes, flow cytometry, catecholamines

INTRODUCTION

It is well known, that the outcome of patients undergoing cardiovascular surgery with cardiopulmonary bypass (CPB) is greatly affected by the induction of a systemic inflammatory response syndrome (SIRS) [1]. Development of SIRS following CPB depends on many different factors: contact of blood with foreign surfaces, ischemia, hypothermia, hypotension, non-pulsatile blood flow and surgical trauma. The administration of catecholamines such as epinephrine (Epi) and norepinephrine (Nor) may additionally act as a modulator of the inflammatory response [2-4].

The dysregulation of the immune response is characterized by an excessive change in the expression of pro- and anti-inflammatory cytokines, growth factors and adhesion molecules [5, 6]. This immunological imbalance is thought to be one of the main inducers of multiorgan dysfunction syndrome (MODS), characterized by the failure of two or more organ systems. The rapid progression from SIRS to sepsis and severe sepsis demands an efficient prevention strategy or effective therapy during the early stage of the syndrome [7]. To develop appropriate and efficient therapeutic approaches, a better understanding of the kinetics of production of proinflammatory cytokines

such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8) is crucial. Recent studies revealed elevated levels of TNF- α , IL-6 and IL-8 during and after CPB [5, 8-10].

TNF- α is a cytokine produced by macrophages, monocytes and other antigen-presenting cells following stimulation with endotoxin. Although it is also required for the normal immune response, an over-expression has severe pathological consequences. As a primary cytokine it plays an important role in the early phase of the immunological response in inflammation and sepsis, where its activity ranges from complement and coagulation cascade activation to upregulation of endothelial adhesion molecules [11]. Furthermore, TNF- α is a potent inducer of fever and hypotension leading to vasoactive shock in humans [11]. IL-6 is secreted by various activated cell types (B- and T-cells, macrophages, fibroblasts, endothelial cells, cancer cells), and is involved in modulating the acute phase response and T-cell activation and differentiation [12].

IL-8 is produced by a number of different cell types, such as stimulated monocytes, macrophages and endothelial cells. IL-8 is chemotactic for all known types of migratory immune cells. It promotes the adhesion of leukocytes to activated endothelial cells and therefore possesses pro-

inflammatory activities [11]. The polypeptide IL-8 is not produced constitutively, but its production is upregulated by other cytokines such as IL-1 α , IL-1 β and TNF- α [13]. Monocytes as a part of the non-specific immune system, are a major source of proinflammatory cytokines such as TNF- α , IL-6 and IL-8 [14]. In infections caused by Gram-negative microorganisms, the principal bacterial constituent recognized by the innate immune system is lipopolysaccharide (LPS), a glycolipid in the outer bacterial membrane [15]. Lipopolysaccharide-binding protein (LBP) initiates the recognition and monomerization of LPS aggregates and its transfer to mononuclear cells. LBP binds to LPS and in low concentrations, catalyzes its transfer to membrane-bound CD14, a glycosylphosphatidyl-inositol (GPI)-linked protein that is part of a cellular receptor for LPS [16]. Interaction with CD14 surface receptor leads to an activation of cytoplasmic transcription factors through the NF- κ B pathway [17].

Catecholamines are administered as vasoactive drugs to patients in cases of difficult weaning from CPB, and to stabilize hemodynamics during postoperative treatment. Epinephrine and norepinephrine are known to influence cytokine expression of whole blood and different cell populations, in a pro- and anti-inflammatory manner [18-22]. This immunomodulating effect has been reported by other authors [23-27]. Few data are known about the time course of intracellular proinflammatory cytokine levels in monocytes obtained by fluorescence-activated cell sorter analysis (FACS), demonstrating the intracellular single cell situation. The aim of our investigation was (1) to establish a validated system of endotoxin-stimulated whole blood as an *ex-vivo* model in order to study cytokine synthesis in inflammatory conditions in which many physiological, cellular interactions remain intact, (2) to test different concentrations of LPS as a model of different stages of inflammation, (3) to compare the effects of the two common vasoactive drugs epinephrine and norepinephrine on the cytokine response of monocytes.

MATERIALS AND METHODS

Subjects

Intravenous blood samples were taken from a pool of nine healthy volunteers and processed immediately under sterile conditions. For each experiment, blood was taken from five subjects chosen from this pool of volunteers. The mean age was 31 ± 8 years. The pool group consisted of two females and seven males. Blood collecting systems comprised of "butterflies" (Winged Infusion System, Becton Dickinson, Sandy, Utah, USA) connected to a 7.5 ml, sterile heparin-prefilled tube (Sodium-Heparin NH/7.5 ml Monovette®, Sarstedt, Nuernbrecht, Germany). Blood was incubated in 5 ml Falcon® polystyrene tubes (Becton Dickinson, Heidelberg, Germany). Diluted and stimulated blood samples were incubated in a 37 °C incubator with a humidified, 5% CO₂ atmosphere.

Setting of experiments

Kinetics of intracellular cytokines in human monocytes

To determine the best time point suitable for the measurement of each cytokine, the kinetics of TNF- α , IL-6 and

IL-8 was investigated. For this reason, 200 μ l of whole blood from five healthy volunteers was stimulated with LPS (10 ng/ml) for 45 min, 1.5 hours (h), 3 h, 6 h and 12 h. Thereafter, samples were processed as described in the section Fluorescence-activated cell sorter analysis (see below).

LPS dose-effect curve for IL-8 and TNF- α

To assess the dose-effect curves for IL-8 and TNF- α , 200 μ l of whole blood from five subjects were incubated with LPS (concentration ranges from 10^{-4} ng/ml to 10 ng/ml) for three hours. This incubation time was chosen because intracellular production of IL-8 and TNF- α reached a maximum at 3 h. After processing the samples for FACS analysis, the percentage of cytokine positive monocytes was assessed. Percent age of positives at 3 ng/ml LPS was taken as 100%.

Effect of epinephrine and norepinephrine on the production of cytokines administered at different times

The effect of Epi and Nor on the production of IL-6, IL-8 and TNF- α was examined and compared. We investigated a possible influence of the administration order of catecholamines and LPS on cytokine production: 200 μ l of whole blood was preincubated for 15 min with Epi (10 μ M) or Nor (10 μ M), followed by stimulation with LPS (1 ng/ml), or catecholamines were administered just before and after LPS (1 ng/ml). The samples were incubated for 3 h. The concentration (10 μ M) of Epi and Nor was chosen according to the papers of Van der Poll [24] and Maes *et al.* [26]. The percentages of cytokine positive monocytes and the mean fluorescence intensities were assessed.

To investigate a possible effect of different catecholamine concentrations on LPS-induced cytokine production, 200 μ l of whole blood (samples from five healthy volunteers) were stimulated with LPS (10 ng/ml) and incubated with Nor and Epi (10 μ M and 100 μ M) for 6 h. Stimulation without catecholamines was taken as 100%.

Influence of Epi and Nor on cytokine kinetics

To examine the possible ability of Epi (10 μ M) and Nor (10 μ M) to influence cytokine production at different time points, blood samples were stimulated with LPS (1 ng/ml) and incubated with Nor and Epi (respectively 10 μ M) for 45 min, 1.5 h, 3 h and 6 h.

Dose-dependent effect of Nor on monocytes stimulated with different LPS concentrations

To investigate the dose-dependent effect of Nor, 200 μ l of whole blood from five subjects was stimulated with different LPS concentrations (0.2 ng/ml, 1 ng/ml, 10 ng/ml) and incubated with Nor (10 μ M and 100 μ M) for 3 h.

Fluorescence-activated cell sorter (FACS) analysis

Two hundred μ l of whole blood was diluted 1:5 with 800 μ l RPMI and 1 μ l GolgiPlug™ in a 5 ml Falcon Tube and vortexed. LPS at different concentrations was used to stimulate monocytes.

After stimulation, cell samples were washed with 1 ml CellWash per tube once, followed by centrifugation at $500 \times g$ for 5 minutes. The pellets were resuspended, washed with 1 ml staining buffer per tube, followed by a centrifugation step. The pellet was resuspended and 10 μ l CD14-FITC antibody was added. The samples were stored at room temperature, in the dark for 10 minutes. Thereaf-

ter, the samples were washed twice with staining buffer. For fixation, 250 μ l Cytofix/Cytoperm per tube was added and incubated at 4 °C, in the dark for 10 minutes.

Thoroughly resuspended, fixed and permeabilized cells (100 μ l) were mixed with 10 μ l per tube of PE-conjugated, anti-cytokine antibody or isotype-control and incubated at 4 °C, for 30 minutes in the dark. Cells were washed twice with Perm/Wash-Buffer and resuspended in 300 μ l of staining buffer.

Flow cytometric analysis was performed using a FACS-Calibur® from Becton Dickinson (Heidelberg, Germany). Monocytes were identified through sidescatter and CD14⁺ labeling. Both cytokine-positive CD14⁺ and mean fluorescence intensities were assessed. Stimulated versus unstimulated samples were used as negative controls.

Reagents

RPMI 1640 (with glutamine) cell culture medium and Dulbeccos-phosphate buffered saline (PBS) were obtained from GIBCO BRL/Life Technologies (Karlsruhe, Germany).

Lipopolysaccharide (LPS) from *E. coli* (055:B5), reconstituted in PBS (1 mg/ml aliquots stored at -80 °C) was purchased from SIGMA (Deisenhofen, Germany).

Brefeldin A (GolgiPlug™) (PharMingen, Becton Dickinson, Heidelberg, Germany) was used as a secretion blocking agent. Fluorescein isothiocyanate (FITC)-conjugated CD14 (PharMingen, Becton Dickinson, Heidelberg, Germany) was used as monoclonal antibody for monocyte identification. Phycoerythrin-(PE) conjugated, anti-human IL-6, IL-8, TNF- α and appropriate isotype controls were purchased from PharMingen (Becton Dickinson, Heidelberg, Germany).

Epinephrine (Epi) and Norepinephrine (Nor) were obtained from Hoechst AG (Frankfurt/Main, Germany).

CellWash, Cytofix/Cytoperm-Solution and Perm/Wash-Buffer were purchased from PharMingen (Becton Dickinson, Heidelberg, Germany). The stain buffer contained PBS (Calbiochem), bovine serum albumin 0.2% (SIGMA, Deisenhofen, Germany), and sodium azide 0.1% (SIGMA, Deisenhofen, Germany).

Statistics

The number of measurements per experiment was $n = 5$. For analysis, cytokine-positive monocytes, as well as mean fluorescence intensities, were assessed. Statistics were conducted using Microsoft Excel. The figures were created with Origin from OriginLab (Northampton, MA, USA). Significant values were determined by the paired Student's t-test to significance of $p < 0.05$ (*) and $p < 0.01$ (**). Data values for cytokine-positive monocytes and mean fluorescence intensities were taken as mean \pm standard error of the mean (SEM).

RESULTS

Kinetics of intracellular cytokines in human monocytes

To determine the best timepoint for measurement of each cytokine, the kinetics of TNF- α , IL-6 and IL-8 were investigated. Figure 1 shows the highest percentage of TNF- α -

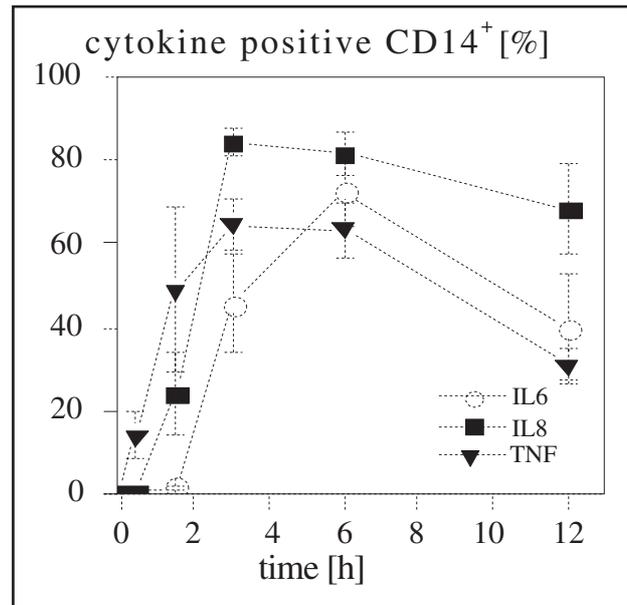


Figure 1

Kinetics of intracellular cytokines in CD14⁺ monocytes. The course of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α cytokine expression was determined by flow cytometry. Monocytes were stimulated with lipopolysaccharide (LPS) (10 ng/ml) for 45 min, 1.5 h, 3 h, 6 h and 12 h. Values are mean \pm standard error of the mean (SEM) of % positives in the CD14⁺ subpopulation.

(64.52 \pm 5.85%) and IL-8- (84.39 \pm 3.06%) positive monocytes after 3 h of stimulation, whereas IL-6 positive monocytes show their peak percentage (72.67 \pm 8.59%) after 6 h of stimulation.

Only TNF- α was detectable at 45 min (14.09 \pm 5.75%) and was the cytokine with the highest expression rate (49.04 \pm 20.02%) at 1.5 h.

IL-8 started to increase significantly at 1.5 h (24.32 \pm 9.93%) and peaked at 3 h (84.39 \pm 3.06%). Afterwards, it started to decrease slightly, but remained at the high level of 68.4 \pm 10.8% IL-8⁺ cells at 12 h.

LPS dose-effect curve for IL-8 and TNF- α

Figure 2 shows that both cytokines had the same dependence upon the LPS concentration. The IL-8 and TNF- α expression in monocytes decreased markedly between 10⁻¹ ng/ml and 1 ng/ml. No effect of LPS stimulation could be recognized at and below concentrations of 0.003 ng/ml. We estimated the LPS ED₅₀ to be 0.2 ng/ml for IL-8 and TNF- α . Therefore, 1 ng/ml LPS was chosen as the stimulation dose for further experiments, to be sure of a maximum stimulation of monocytes still being effect by catecholamines.

Effect of epinephrine and norepinephrine on the production of cytokines administered at different times

Table 1a shows that preincubation with Epi (10 μ M) and Nor (10 μ M) for 15 min, compared to treatment simultaneously with LPS (1 ng/ml) application, had no effect on cytokine production by monocytes. Catecholamine administration before and after the LPS stimulus had no effect on the percentage of cytokine-positive monocytes.

After 3 h of LPS stimulation, no significant difference in cytokine expression due to Nor and Epi could be demon-

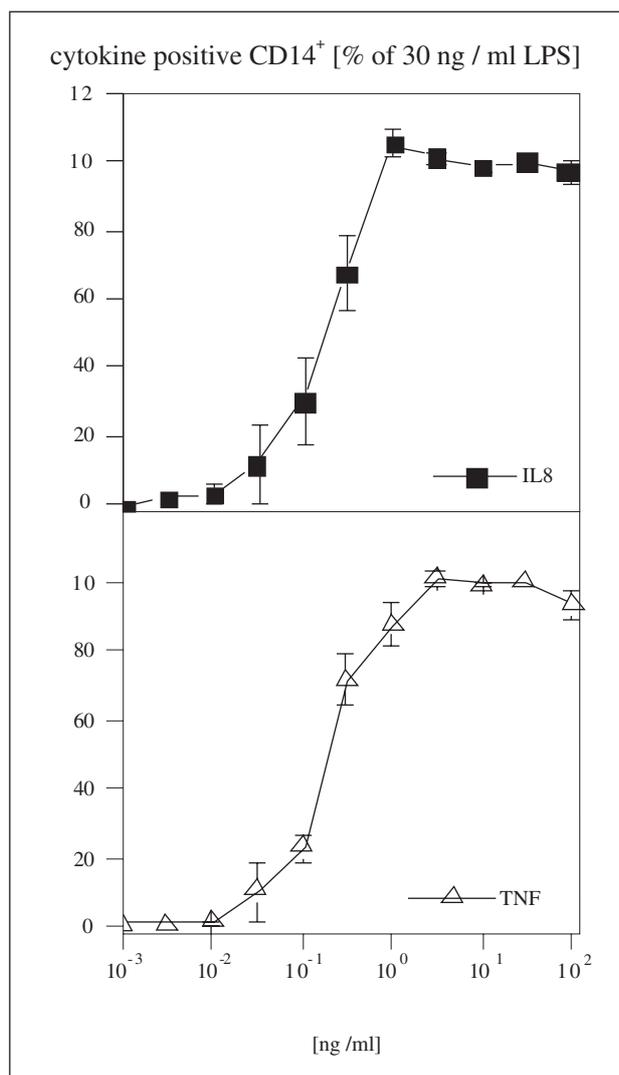


Figure 2

IL-8- and TNF- α -positive CD14⁺ positive cells after 3 h of stimulation with lipopolysaccharide (LPS) at different concentrations in whole blood samples. Values are mean \pm standard error of the mean (SEM) of percentage positives in the CD14⁺ subpopulation.

strated. The percentage of IL-6-positive CD14⁺ cells were decreased significantly ($p < 0.05$) by Nor and Epi. TNF- α -positive CD14⁺ were inhibited more than IL-6 positive CD14⁺. The number of IL-8-positive CD14⁺ remained over 90%, and was not affected at 3 h (Table 1a).

After 6 h of stimulation, only TNF- α -positive CD14⁺ cells were significantly decreased by Epi and Nor ($p < 0.01$).

Table 1a

Blood from five healthy subjects was stimulated with LPS (1 ng/ml) for 3 hours. Epi (10 μ M) and Nor (10 μ M) were administered 15 minutes before, directly before and directly after LPS stimulus. Values are mean \pm standard error of the mean (SEM) of percentage positives in the CD14⁺ subpopulation. * $p < .05$; ** $p < .01$ versus control

LPS	Epi			Nor		
	IL-6	IL-8	TNF- α	IL-6	IL-8	TNF- α
Without	80.77 \pm 1.65	91.22 \pm 2.69	89.76 \pm 1.95	80.77 \pm 1.65	91.22 \pm 2.69	89.76 \pm 1.95
15 min preincubation	66.57 \pm 3.58*	93.14 \pm 2.42	61.30 \pm 3.86*	64.67 \pm 4.25	94.17 \pm 1.53	55.98 \pm 9.65*
Catecholamine before LPS	71.66 \pm 1.62**	93.61 \pm 0.99	64.86 \pm 4.49*	69.48 \pm 4.49	92.89 \pm 1.64	63.11 \pm 3.28*
Catecholamine after LPS	70.36 \pm 1.16*	93.17 \pm 1.89	67.86 \pm 4.14*	66.66 \pm 3.48*	94.34 \pm 1.56	67.27 \pm 4.05*

IL-6- and IL-8-positive CD14⁺ were unaffected at 6 h. Catecholamine incubation without LPS showed no effect on IL-6, IL-8 and TNF- α production (data not shown).

When assessing mean fluorescence intensities for IL-6, IL-8 and TNF- α in the same setting (Table 1b), the results, according to the influence of Epi and Nor and the administration order on cytokine expression, were the same compared with data analysis of the percentage of cytokine-positive cells.

Effect of Epi and Nor, administered at different timepoints, on cytokine expression

The effect of different catecholamine concentrations (10 μ M and 100 μ M) on LPS-induced cytokine production is demonstrated in Figures 3a and 3b. Stimulation without catecholamines was taken as 100%.

Figure 3a shows that IL-6⁺ monocytes were not significantly inhibited by 10 μ M and 100 μ M Epi. There was a significant inhibition of 78.1 \pm 3.47% when Nor was administered at concentrations of 100 μ M.

IL-8 production was unaffected by Epi and Nor at both concentrations (Figure 3a).

TNF- α production was affected in a dose-dependent manner, which was highly significant ($p < 0.01$) for both concentrations (Figure 3a). It was reduced to 68.13 \pm 5.93% at 10 μ M, and to 39.05 \pm 7.52% at 100 μ M Nor. The same results could be found for Epi: TNF- α production was reduced to 75.4 \pm 3.33% by Epi (10 μ M) and to 46.77 \pm 7.05% by Epi (100 μ M). No difference in the effect of either catecholamine was detected (data not shown).

The percentage of monocytes (8.6 \pm 0.6%), which was comparable to its fraction in the white cell blood count, did not change during incubation with Epi and Nor at any concentration, thus excluding a decrease of cytokine-positive cells resulting from a possible toxic effect of these catecholamines.

By assessing the mean fluorescence intensities for the same experiment, the general, inhibiting effect of TNF- α production by Epi and Nor, in a dose-dependent manner could be confirmed (Figure 3b). Differences, compared to the data analysis for cytokine-positive cells, were that the reduction of IL-6 production by 10 μ M Epi became significant, and that IL-8 production was inhibited by Nor (10 μ M) to 79.84 \pm 2.1%.

Influence of Epi and Nor on cytokine kinetics

Figure 4 demonstrates the time course of the effects of Epi (10 μ M) and Nor (10 μ M) on cytokine production after LPS stimulation (final concentration 1 ng/ml), and differ-

Table 1b
Cytokine staining (mean fluorescence intensity) of CD14⁺ -positive monocytes after stimulation with LPS (1 ng/ml) for 3 hours. Epi (10 μM) and Nor (10 μM) were administered 15 minutes before, directly before and directly after LPS stimulus. Values are mean ± standard error of the mean (SEM). *p < .05; **p < .01 versus control

LPS	Epi			Nor		
	IL-6	IL-8	TNF-α	IL-6	IL-8	TNF-α
Without	19.15 ± 0.32	28.47 ± 2.49	43.75 ± 6.90	19.15 ± 0.32	28.47 ± 2.49	43.75 ± 6.90
15 min preincubation	15.62 ± 0.68*	29.09 ± 1.85	19.41 ± 1.40*	15.00 ± 0.55*	30.20 ± 2.07	24.39 ± 7.02
Catechecholamine before LPS	16.64 ± 0.08*	30.38 ± 2.16	25.50 ± 1.66*	16.01 ± 0.16*	30.03 ± 2.25	24.35 ± 1.65*
Catecholamine after LPS	16.26 ± 0.34*	30.40 ± 2.52	26.09 ± 1.22*	15.72 ± 0.78*	30.77 ± 1.82	25.52 ± 2.53*

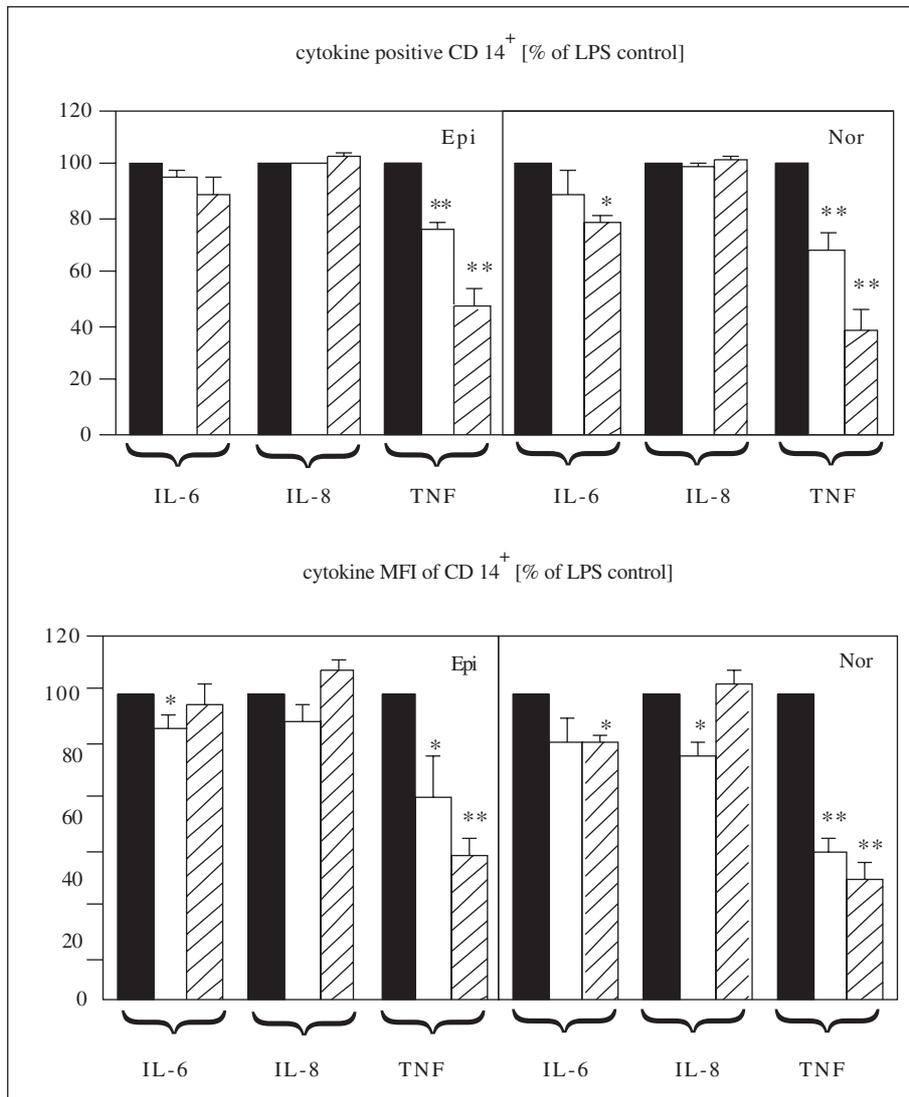


Figure 3

Effect of noradrenaline (Nor) and epinephrine (Epi) on the intracellular IL-6, IL-8 and TNF-α expression in CD14⁺ positive monocytes after 6 h of stimulation with LPS (10 ng/ml). Filled bars show the LPS control. Open bars show 10 μM catecholamine and crossed bars show 100 μM catecholamine. (a) Values are percentage positives in the CD14⁺ subpopulation based on the LPS control (without Nor or Epi) taken as 100%. Data are mean ± standard error of the mean (SEM). *p < .05; **p < .01 versus control. (b) Cytokine staining (mean fluorescence intensity) of CD14⁺ -positive monocytes after stimulation with LPS and incubation with Epi and Nor. Values are mean ± standard error of the mean (SEM). *p < .05; **p < .01.

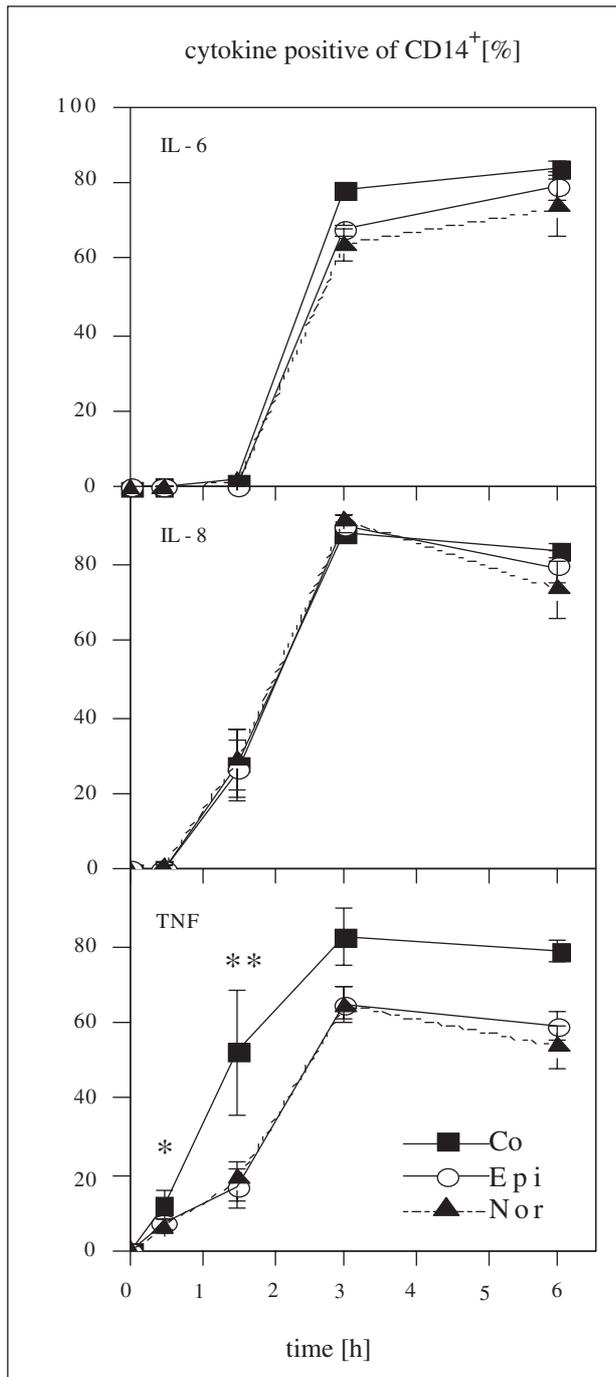


Figure 4

Effect of Nor (10 μ M) and Epi (10 μ M) on cytokine expression of human monocytes detected at different time points (45 min, 1.5 h, 3 h, 6 h) after stimulation with (LPS) (1 ng/ml). Values are mean \pm standard error of the mean (SEM) of percentage positives in the CD14⁺ subpopulation. * p < .05; ** p < .01.

ent incubation times (45 min, 1.5 h, 3 h and 6 h). The inhibition of IL-6⁺ CD14⁺ cells by Nor and Epi showed no significant difference: IL-6-producing CD14⁺ were slightly reduced after 3 h of incubation, from $77.95 \pm 1.73\%$ (control) to $67.54 \pm 1.36\%$ with Epi, and to $63.85 \pm 4.07\%$ with Nor (p < 0.05).

There was no effect on IL-8 production with either catecholamines (Figure 4).

TNF- α was influenced by Epi and Nor between 45 min to 6 h of incubation. At 45 min, TNF- α production was significantly (p < 0.05) reduced from $12.44 \pm 3.93\%$ TNF- α ⁺ (control) to $7.65 \pm 2.12\%$ TNF- α ⁺ (Epi), and to $6.34 \pm 1.83\%$ TNF- α ⁺ (Nor). At 1.5 h, the inhibiting effect was even more evident: $52.27 \pm 16.53\%$ TNF- α ⁺ (control) to $16.58 \pm 4.79\%$ TNF- α ⁺ (Epi), and to $18.69 \pm 5.18\%$ TNF- α ⁺ (Nor).

At 3 h and 6 h, the inhibiting effects on TNF- α were not as distinctive as at 1.5 h: The reduction at 3 h was 21.06% for Epi and 21.78% for Nor. At 6 h, Epi reduced TNF- α expression by 25.39% and Nor reduced this expression by 31.88%.

Dose-dependent effect of Nor on monocytes stimulated with different LPS concentrations

The effect of Epi and Nor on monocytes stimulated with low doses of LPS (0.2 ng/ml) for 3 h is shown in comparison to a submaximal (1 ng/ml) and maximal LPS stimulus (10 ng/ml) in Figure 5. Following the addition of 10 μ M Nor, the percentage of IL-6⁺ monocytes decreased by 24.93% with 10 ng/ml LPS, by 32.77% with 1 ng/ml LPS and by 58.25% with 0.2 ng/ml LPS, compared to the control. The inhibiting effect of Nor was stronger with lower concentrations of LPS. The difference between the controls and the varying LPS concentrations, and the values for LPS 1 ng/ml to 0.2 ng/ml were significant (p < 0.05).

IL-8⁺ monocytes were not significantly influenced by 10 μ M Nor. At 100 μ M Nor, decreases to $93.33 \pm 2.04\%$ (LPS = 10 ng/ml), to $83.96 \pm 3.44\%$ (LPS = 1 ng/ml) and to $43.11 \pm 3.34\%$ (LPS = 0.2 ng/ml) were detected (Figure 5).

TNF- α ⁺ CD14⁺ cells were inhibited in the same way as IL-6 for Nor (10 μ M). The percentage of TNF- α CD14⁺ cells was reduced to 42.70 ± 5.27 (LPS 10 ng/ml), to $34.80 \pm 4.02\%$ (LPS 1 ng/ml), to $19.02 \pm 3.13\%$ (LPS 0.2 ng/ml). Maximum inhibition was observed with the lowest concentration of LPS, with a decrease of 80.98% of TNF- α ⁺ cells. The same results were found for Nor (100 μ M), with a stronger inhibitory effect. The values of TNF- α for the LPS concentrations differed significantly (p < 0.05) from the controls. The difference between LPS 1 ng/ml and LPS 0.2 ng/ml was significant (p < 0.05).

TNF- α production was most influenced by Nor (100 μ M), at very low LPS doses.

DISCUSSION

In cardiothoracic surgery, SIRS and sepsis occur more often than after other operations with equivalent trauma [29]. CPB is associated with ischemia and reperfusion injury to the heart and lungs and also remote organs such as the kidney, gut and the brain. This organ damage may lead to translocation of bacterial antigen to the circulation, inducing a significant inflammatory response. Escalation from inflammation to "severe SIRS" may be initiated by cytokines, which are produced by various immune competent cells. Monocytes are considered to be a major source of proinflammatory cytokines [14]. The cytokines chosen for this study play a major role in the pathogenesis of sepsis and SIRS, and are suggested to be markers of the

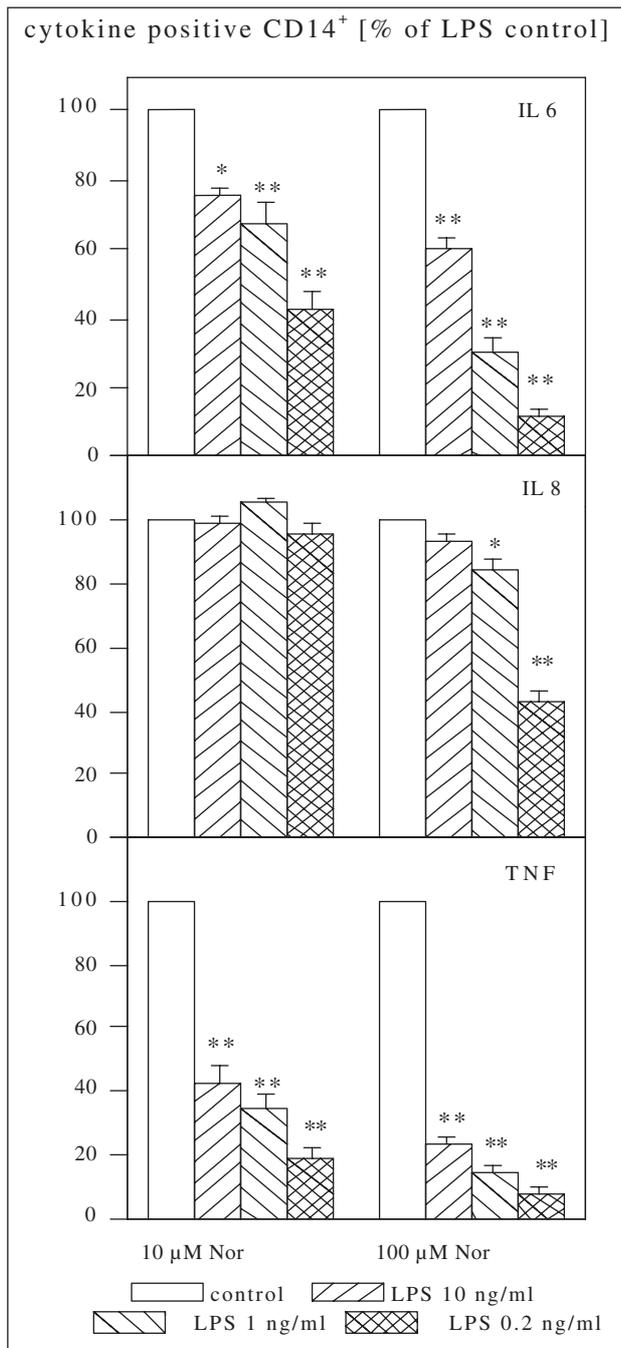


Figure 5

Comparative and interactive effects caused by different lipopolysaccharide (LPS) concentrations and noradrenalin concentrations (10 μM and 100 μM) on interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α cytokine expression in monocytes. Controls were taken as 100%. Data are mean ± standard error of the mean (SEM) of percentage positives in the CD14⁺ subpopulation. **p* < .05; ***p* < .01 versus control.

outcome for critically ill patients [11, 30, 31]. Proinflammatory cytokines such as IL-6, IL-8 and TNF-α particularly play a pivotal role in the pathogenesis of inflammation, and are involved in the initiation of MODS following cardiovascular surgery [10]. The influence of catecholamines on proinflammatory cytokine production has been reported previously [19, 21, 22, 27, 32]. Adrenergic amines that are able to increase intracellular cAMP levels, like Epi and Nor, have been shown to be potent suppressors

of the pro-inflammatory cytokine release from monocytes [18, 19, 32]. Knowing how catecholamines influence the immune function might be of great interest, because cardiothoracic patients are often treated with positive inotropic agents to maintain the hemodynamic balance after CPB. A differentiated view on immune modulating substances is needed because the mortality from sepsis remains very high, despite the efforts with immune modulating therapies in recent years [33, 34]. In this study, a whole blood *ex vivo* system was used to investigate the monocyte immune response to LPS under the influence of Nor and Epi. The cytokine release was compared for both catecholamines, at two different concentrations.

In order to investigate the kinetics of intracellular IL-6, IL-8 and TNF-α expression, it was necessary to determine the best time point for comparing the effects of catecholamines on monocyte cytokine production. IL-6, IL-8 and TNF-α expression in monocytes was highest between 3 h and 6 h after stimulation with LPS. Various studies have reported on the different cytokine kinetics and the effects of catecholamines on monocytes [18]. Most groups used the ELISA technique for measuring cytokines [18]. The methodical problem using ELISA in a whole blood system is that the cytokine concentration in the supernatant is an integrated quantity of different cell populations [35]. On the other hand, cell culture systems in which single cell populations are examined lack the involvement of possible co-factors, which modulate the stimulation process. Intracellular FACS analyses allow an individual characterisation of large cell populations and thanks to multi-colour staining, it exclusively allows cell surface phenotyping and characterization of cytokine expression in heterogenic cell suspensions such as whole blood [36, 37].

The significant increase in cytokine production due to LPS stimulation observed in the present study started at 45 min for TNF-α and IL-8 and at 1.5 h for IL-6. This is in contrast to the results of Kwak *et al.*, who described an increase in TNF-α production after 4 h, in response to an LPS stimulus [37]. The peaks of cytokine expression were different, too. Kwak *et al.* found peaks for all three cytokines after 4 hours. In our study, the peak for TNF-α was at 3 h; IL-8 and IL-6 peaked at 6 h. One of the reasons for these differences could be that different LPS serotypes in different concentrations were used. The method involved in has monocytes are processed may also influence the results: the isolation of monocytes through density-gradient centrifugation, followed by incubation without possible co-factors, could explain the differences between both studies.

Lin *et al.* investigated the production of pro-inflammatory cytokines *in vivo* using the ELISA technique [38]. Administering a bolus injection of 2 ng/kg of LPS in five healthy humans, they found a similar expression sequence for TNF-α and IL-6. IL-8 was found to be expressed later than IL-6 and TNF-α. IL-8 was the most highly expressed cytokine in our study. Lin *et al.* [38] found IL-8 to be expressed at a low level compared to TNF-α and IL-6. Differences in cytokine kinetics could be explained because Lin *et al.* were not investigating monocyte subsets in their whole blood *in vivo* system. These findings indicate that *in vitro* findings have to be critically assessed for their transfer *in vivo*. Furthermore, individual cytokine characteristics and formation of the immune competent cells that they descend from have to be taken into account. The next

question was to determine the ED₅₀ LPS concentration for further monocyte stimulation, and to compare these findings with clinical situations. Cytokine production was obtained with LPS concentrations ranging from 10² ng/ml to 10⁻³ ng/ml. The ED₅₀ for IL-8 and TNF- α (Figure 2) in our whole blood system was determined to be at 0.2 ng/ml LPS. In other studies, higher LPS concentrations were used [39-41]. Ertel *et al.* [40] used 1 μ g/ml of LPS to get a maximal cytokine expression, and 1 ng/ml of LPS, a concentration while can be reached under clinical conditions. We could show that LPS concentrations higher than 1 ng/ml did not lead to a further increase in intracellular cytokine expression in human monocytes. LPS at 1 ng/ml was chosen for our experiments because the influence of catecholamines should not be masked by a possible monocyte overstimulation as a result of an excessive LPS concentration. In septic patients (gram negative origin), an LPS concentration of 1 ng/ml was found to be appropriate by Shenep *et al.* [42] and Van Deventer *et al.* [43]. After evaluation of these findings altogether, we decided not to use LPS concentrations higher than 10 ng/ml in further experiments.

Although the suppressive effect of adrenergic amines is suspected to be mediated by different receptors, as reported for beta-receptors [19] and alpha₂-receptors [44], no difference between the effect of Epi and Nor on the expression of IL-6, IL-8 and TNF- α in monocytes could be shown (Table 1 and 2). Both catecholamines were able to inhibit the IL-6 and TNF- α expression, although TNF- α more than IL-6. IL-8 was unaffected even at 100 μ M Nor and Epi (Figure 3a and b). These findings are generally in agreement with other studies, which showed the same effect for IL-6 and TNF- α [19, 22]. Van der Poll *et al.* [22] have found a dose-dependent inhibition of Nor on IL-6 in a whole blood system. Whole blood was incubated for 4 h with 1 ng/ml and 10 ng/ml LPS. Nor was administered with final concentrations of 10⁻⁹ to 10⁻⁶ M. Cytokine expression was measured by ELISA. IL-6 expression was inhibited significantly at Nor 10⁻⁵ and 10⁻⁶ M. These findings correspond to the findings in our present study in which IL-6 was inhibited by Nor (10 μ M) and significantly by 100 μ M Nor (Figure 3a and b). In addition we found that Epi showed a significant inhibiting effect at 10 μ M (Figure 3b).

Other investigations of van der Poll *et al.* [45] revealed that Epi decreases TNF- α expression in a dose-dependent fashion. Whole blood was stimulated with LPS at 10 ng/ml and incubated with Epi (final concentrations ranged from 10⁻⁸ and 10⁻⁵ M), for up to 16 h. TNF- α expression was assessed by ELISA. A maximum inhibiting effect was found at 10⁻⁶ M Epi. These results are in accordance with our data, where Epi (10 μ M and 100 μ M) attenuates intracellular TNF- α expression (Figure 3a and b).

Figure 4 shows that Epi (10 μ M and 100 μ M) inhibited TNF- α expression at up to 6 h of incubation. While the inhibiting effect assessed by van der Poll *et al.* [45] was 80%, we found an inhibition of 25% after 6 h of incubation (Figure 4). This might be explained by the assumption that other cell types may be involved *in vivo*, resulting in a greater inhibitory effect of Epi.

Van der Poll *et al.* examined the effect of Epi *in vivo* [45]. TNF- α production was investigated after stimulation with LPS under the influence of a constant Epi infusion. Blood samples were taken directly before infusion and 4 h, 8 h,

21 h and 24 h after starting the Epi infusion. Plasma levels of TNF- α were attenuated by Epi from the 4 h time point over the whole infusion period. In the present study we showed that intracellular TNF- α expression in human monocytes was affected earlier (45 min), and remained at decreased level (Figure 4).

For IL-8, the effect of cAMP-elevating drugs is reported in different ways. Farmer *et al.* [46] found a decrease under the influence of isoproterenol, and Kavelaars *et al.* [20] found an increased IL-8 production in response to beta₂-receptor stimulation. In our study, we found that IL-8 was not influenced significantly by Epi or Nor. IL-8 expression was investigated by van der Poll *et al.* [21] under the influence of Epi. IL-8 expression was assessed by ELISA in a whole blood system. Blood was incubated with LPS (10 ng/ml) in the presence and absence of Epi (10⁻⁸ to 10⁻⁵ M). A dose dependent increase in IL-8 expression was shown with significant results when Epi (10⁻⁷ to 10⁻⁵ M) was used. IL-8 expression was increased significantly after 4 h. Compared to these results, data from our investigation with 1 ng/ml LPS stimulation and incubation with Epi (10 μ M) and Nor (10 μ M) for different time intervals reveal that intracellular IL-8 expression in monocytes is not affected at any time up to 6 h (Figure 4). This difference may be caused by the fact that monocytes as a subset were not assessed in the study of van der Poll *et al.* [21] and that other immune competent cells were probably involved in the resulting IL-8 plasma levels. Van der Poll *et al.* showed a maximum increase of IL-8 expression after 24 h incubation with Epi.

Kavelaars *et al.* [20] found an increase in IL-8 after 8 h of LPS incubation, with a maximum increase after 18 hours of incubation. Farmer *et al.* [46] stimulated for 8 h. These findings taken together lead to the conclusion, that in this present study, no significant effect of Epi and Nor could be observed, because the incubation time was too short. We assume that the activated signal transduction cascades for IL-8 expression are activated at maximum, so that the effect of elevated cAMP is not strong enough to influence IL-8 expression. These results for IL-8 underline the fact that incubation time is of great importance when investigating the influence of catecholamines on cytokine expression in monocytes.

No difference in the inhibition of cytokine expression was detected for Nor and Epi. This may be explained by the fact that the occupation of beta and alpha receptors by Nor and Epi depends on the concentration of catecholamine in the sample. We presume, that at high concentrations of Nor and Epi, the same adrenergic receptors or the ratio of the different receptor subtypes are occupied, resulting in an identical effect. Concentrations of 10 μ M and 100 μ M of Epi and Nor were chosen for maximum effect and to obtain comparable results to other studies [28]. These concentrations do not represent a clinically relevant situation, but we could show that the inhibiting effect of 10⁻⁵ M Nor was the same as 10⁻⁷ M Nor for TNF- α and IL-8. Grundman *et al.* [47] measured a concentration of 10⁻⁸ M Nor in patients 20 min after CPB. We suppose that the clinical concentrations of Nor, as reported by Grundman *et al.*, have an effect on pro-inflammatory cytokine production in monocytes.

Cytokines such as IL-6, IL-8 and TNF- α are mandatory mediators in the onset of an immune response to bacterial antigen in humans. Many patients who have undergone

CPB or who suffer from other stressors, have elevated Nor and Epi plasma levels, which may influence the immune response [47, 48]. Under these circumstances, the susceptibility to bacteria might be increased. Figure 5 shows that Nor impairs cytokine expression more if the LPS stimulus is not of maximum strength. These results lead to different assumptions: on the one hand, monocytes, as one of the first players of the immune system coming in contact with antigen in the blood may not be properly activated, resulting in possible mediator paralysis, with an increase in virulence of the antigen. On the other hand, a protective effect from excessive cytokine production, which is associated with MOF and MODS may be positively influenced through catecholamines, indicating a possible therapeutic option or at least, a positive, additive effect.

In this study, all of the experiments were performed with whole blood from healthy volunteers. Transposing the results to the pathophysiological situation *in vivo* has to be critically discussed. Particularly after CPB and in severe sepsis, the immunological response to endotoxin from Gram negative bacteria is altered. Wilhelm *et al.* [49] found a deactivation of TNF- α expression 20 min after CPB and within the first eight days of severe sepsis. This suggests that the effect from catecholamines here might be different compared to the effect observed in whole blood from healthy humans. Additive effects resulting in more pronounced mediator attenuation or the induction of anti-inflammatory cytokines, such as IL-10, may influence the catecholamine-induced alteration of the immune function. Of concern is that Wilhelm *et al.* [49] were investigating patients with severe sepsis who were already being treated with catecholamines, the study being aimed at the assessment of the resulting situation after therapy and not at the pathogenesis of cytokine inhibition by catecholamines. In the pathogenesis of SIRS after CPB, leukocyte desensitization with an impaired ability of monocytes to express pro-inflammatory cytokines is observed. Grundmann *et al.* [47] found Nor to be a humoral factor which is thought to be involved in leukocyte desensitization. In the present study, Nor and Epi inhibited cytokine expression in monocytes, suggesting that the cytokine attenuation by Nor might cooperate with other immunomodulating factors leading to SIRS changing the immune mediator network which might result from CPB.

In conclusion, Epi and Nor show no difference in their inhibitory effect upon proinflammatory cytokine expression in monocytes in response to LPS. At low LPS concentrations, monocyte cytokine expression is more diminished than after stimulation with high LPS concentrations.

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